Hepatitis Delta Virus facilitates the selection of Hepatitis B Virus mutants *in vivo* and functionally impacts their replicative capacity *in vitro*

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Abstract

To identify molecular interactions between hepatitis B virus (HBV) and hepatitis delta virus (HDV), HBV sequences were analyzed in HBV/HDV-infected patients. Characteristic amino acid substitutions were found in cytosolic domains of HBsAg, in contrast to HBV mono-infected controls. The functional impact of HDV on replication of wild-type and mutant HBV was assessed *in vitro*. HDV co-transfection significantly reduced replication of HBV strains containing precore or basal core promoter mutations, while HBV polymerase or surface antigen mutants affected HDV replication *in vitro*. Conclusively, our study revealed distinct HBsAg mutational patterns in HBV/HDV-infected patients and novel functional interactions between HBV and HDV.

Key words: Hepatitis B virus, hepatitis D virus, precore, basal core promoter, HBsAg

Hepatitis D virus (HDV) co- or super-infection in hepatitis B virus (HBV) infected individuals is the most severe form of chronic hepatitis. Although HBV-DNA is oftentimes suppressed in co-infected patients, fluctuating or persistently high levels of HBV viremia occur in delta hepatitis [1, 2]. We recently reported the distinct mutational pattern in the HDV genome from a nationwide Iranian cohort, where the prevalence of HDV is high [3]. We now analyzed the impact of HDV infection on the genomic sequences and functional properties of HBV in double-infected individuals. We studied HBV sequences of S-HBsAg, reverse transcriptase (rt), transcription regulators (including EnhI, EnhII and BOX-β), basal core promoter (BCP) and precore (PC) domains from HBV/HDV-infected patients with amplified HBV-DNA in a case-control setting. For each case with amplified HBV in HBV/HDV-infected patients, two HBV-mono-infected patients matched for sex, age, genotype and HBeAg status were studied as controls. The HBV/HDV infected patients more frequently had cirrhosis, while their HBV-DNA levels were lower than in HBV mono-infected individuals (**Table 1**). Due to reduced HBV-DNA levels in HDV-infected individuals [1], HBV amplification yielded 17 nucleotide sequences for rt/HBsAg and EnhI and 9 sequences for EnhII, Box-β and BCP domains from the total cohort of 71 HBV/HDV infected patients.

To identify hot spots for HDV-driven HBV mutations, the number of non-synonymous and synonymous substitutions per site (dN/dS) for each codon of HBsAg and rt domains was calculated. This analysis revealed a trend towards positive selection of amino acids (increased dN/ds ratio) at cytosolic loop (CYL)-I (for case group) and CYL-II (for case and control groups) of HBsAg (**Fig.1A**), where interactions between HBsAg and HDV ribonucleoprotein take place [4, 5].

We also observed a high rate of amino acid changes in the RT1 (G, F), A and C domains of rt sequences in HBV/HDV-infected patients (**Fig.1A**). While mutations

in the C domain (overlapping with CYL-II in HBsAg) mainly occur in response to antiviral treatments, the RT1 and A changes are presumably related to HBV-RNA priming, packaging and dNTP/NTP discrimination [6, 7].

The striking differences in HBsAg-CYLs/RT domains between HBV-mono- and HBV/HDV-infected patients in contrast to the conserved structure of the HBsAg antigenic loop in both groups strongly indicated functional effects of HDV on HBV replication. We thus comprehensively analyzed the impact of HDV cotransfection on clinically common HBV RT and HBsAg mutants using transient transfection of Huh7 hepatoma cells by replication-competent constructs [8].

In agreement with our previous reports [9, 10], lamivudine-resistant polymerase mutations (SM: rtM204I, DM: rtL180M+rtM204V), which also induce changes at CYL-II of the overlapping HBsAg, as well as their combination with immune escape mutations (sG145R, sP120T), located in the Ag-loop of HBsAg, impaired HBV replication *in vitro*, as evidenced by intra- and extra-cellular levels of HBV-DNA (**Fig.1B-C**). Co-transfection with HDV had overall no major impact on the replication of HBV polymerase or immune escape mutants (**Fig.1B-C**), except for a reduction of intracellular HBV levels of rtM204I, an HBV mutation that potentially inhibits HDV encapsidation [4].

Nearly half of the HDV-infected patients showed two mutations in the HBV-EnhI domain (**Fig.2A**, nucleotides 1131 and 1167). Interestingly, the interaction of HDAg with HBV-EnhI and EnhII may trans-repress HBV replication [11]. Therefore, the altered sequence of EnhI in these patients possibly protects HBV from suppressive effects of HDAg. Further studies are required to confirm the frequency of these mutations in HDV-infected patients and their role in HBV-HDV interactions.

Although all patients were HBeAg-negative, BCP mutations (A1762T/G1764A) were less frequent in HBV/HDV-infected compared to HBV-mono-infected patients (**Fig.2A**, 11% vs. 44%, respectively). These data are in line with previous studies indicating a low prevalence of PC and BCP mutations in HBV/HDV-infected patients [12-14]. On a functional level, BCP-containing mutants showed an increased HBV replication *in vitro* (**Fig.2B-C**), in agreement with previous studies [8]. On the contrary, co-transfection of HBV mutants with HDV significantly reduced the replication of PC- or BCP-mutant HBV constructs (**Fig.2B-C**). These data suggest inhibitory effects of HDV on HBV PC/BCP mutants. In line, low levels of HBV-DNA in both serum and liver of HDV-infected patients with BCP or PC mutations had been reported [12, 13, 15]. Due to the inhibitory binding of HDAg to HBV-Enh elements [11] and the trans-activation of HBV pre-S and S promoters by L-HDAg [16], it is possible that BCP and PC mutations provide new interaction sites for HDV proteins to bind and exert inhibitory effects, for example, by changing transcription factor binding. Moreover, BCP also affects the sequence of the overlapping HBx protein, which cross-talks with L-HDAg in co-infected cells [8, 17].

Next, we studied the effects of different HBV mutations on the replication and secretion of HDV *in vitro*. Mutations in CYLs are considered unfavorable for HDV secretion or infectivity, but tolerable for HBV subviral particle formation; thus, HBV isolates with these mutations are probably selected, when HDV co-replicates [4, 5]. Our *in vitro* experiments indicated a significant decrease of intracellular HDV replication, but increase in HDV secretion in sG145R or sP120T HBsAg-mutations (**Fig.2D-E**). One explanation for this increase might be the cytoplasmic positioning of non-glycosylated-Ag loop of HBsAg molecules [18], which provides an interaction opportunity for HDV RNP [19]. Moreover, due to the proximity of sG145 and sP120 with glycosylation sites of the S-protein, mutations at these residues may affect

lateral S-S interactions, which occur differently for HBV and HDV due to their particle sizes [20]. Notably, PC or BCP mutations did not impact HDV replication or secretion *in vitro* (**Fig.2D-E**).

In conclusion, our study indicates different selective adaptations in the HBV genome induced by HDV, including amino acid changes at interaction sites with HDV and selection of wild-type PC and BCP domains, likely to escape inhibitory effects of HDV on its replication. However, additional factors such as enhanced immune clearance prompted by the dual infection or HDV-induced host cell factors, which interfere with viral replication, need to be considered [16]. Future studies should also include longitudinal analyses in order to unravel the evolution of alterations in the HBV genome in delta hepatitis patients.

References

[1] Hughes SA, Wedemeyer H, Harrison PM. Hepatitis delta virus. Lancet 2011;378:73-85.

[2] Shirvani-Dastgerdi E, Tacke F. Molecular interactions between hepatitis B virus and delta virus. World J Virol 2015;4:36-41.

[3] Shirvani-Dastgerdi E, Amini-Bavil-Olyaee S, Alavian SM, Trautwein C, Tacke F. Comprehensive analysis of mutations in the hepatitis delta virus genome based on full-length sequencing in a nationwide cohort study and evolutionary pattern during disease progression. Clin Microbiol Infect 2014.

[4] Vietheer PT, Netter HJ, Sozzi T, Bartholomeusz A. Failure of the lamivudine-resistant rtM204I hepatitis B virus mutants to efficiently support hepatitis delta virus secretion. J Virol 2005;79:6570-6573.

[5] Komla-Soukha I, Sureau C. A tryptophan-rich motif in the carboxyl terminus of the small envelope protein of hepatitis B virus is central to the assembly of hepatitis delta virus particles. J Virol 2006;80:4648-4655.

[6] Badtke MP, Khan I, Cao F, Hu J, Tavis JE. An interdomain RNA binding site on the hepadnaviral polymerase that is essential for reverse transcription. Virology 2009;390:130-138.

[7] Wang YX, Luo C, Zhao D, Beck J, Nassal M. Extensive mutagenesis of the conserved box E motif in duck hepatitis B virus P protein reveals multiple functions in replication and a common structure with the primer grip in HIV-1 reverse transcriptase. J Virol 2012;86:6394-6407.

[8] Tacke F, Gehrke C, Luedde T, Heim A, Manns MP, Trautwein C. Basal core promoter and precore mutations in the hepatitis B virus genome enhance replication efficacy of Lamivudine-resistant mutants. J Virol 2004;78:8524-8535.

[9] Amini-Bavil-Olyaee S, Vucur M, Luedde T, Trautwein C, Tacke F. Differential impact of immune escape mutations G145R and P120T on the replication of lamivudine-resistant hepatitis B virus e antigen-positive and -negative strains. J Virol 2010;84:1026-1033.

[10] Tacke F, Shirvani-Dastgerdi E. Impact of Drug-Resistance Polymerase Mutations on the Replication of HBeAg-Positive and HBeAg-Negative Hepatitis B Virus Strains in Vitro. Hepat Mon 2012;12:357-360.

[11] Williams V, Brichler S, Radjef N, Lebon P, Goffard A, Hober D, et al. Hepatitis delta virus proteins repress hepatitis B virus enhancers and activate the alpha/beta interferon-inducible MxA gene. J Gen Virol 2009;90:2759-2767.

[12] Brunetto MR, Giarin MM, Rosina F, Smedile A, Mangia A, Oliveri F, et al. Relations between hepatitis delta virus pathogenicity and hepatitis B virus heterogeneity. Prog Clin Biol Res 1993;382:213-219.

[13] Yeh CT, Chiu CT, Tsai SL, Hong ST, Chu CM, Liaw YF. Absence of precore stop mutant in chronic dual (B and C) and triple (B, C, and D) hepatitis virus infection. J Infect Dis 1994;170:1582-1585.

[14] Jardi R, Rodriguez F, Buti M, Costa X, Cotrina M, Galimany R, et al. Role of hepatitis B, C, and D viruses in dual and triple infection: influence of viral genotypes and hepatitis B precore and basal core promoter mutations on viral replicative interference. Hepatology 2001;34:404-410.

[15] Pollicino T, Raffa G, Santantonio T, Gaeta GB, Iannello G, Alibrandi A, et al. Replicative and transcriptional activities of hepatitis B virus in patients coinfected with hepatitis B and hepatitis delta viruses. J Virol 2011;85:432-439.

[16] Wei Y, Ganem D. Activation of heterologous gene expression by the large isoform of hepatitis delta antigen. J Virol 1998;72:2089-2096.

[17] Goto T, Kato N, Yoshida H, Otsuka M, Moriyama M, Shiratori Y, et al. Synergistic activation of the serum response element-dependent pathway by hepatitis B virus x protein and large-isoform hepatitis delta antigen. J Infect Dis 2003;187:820-828.

[18] Werr M, Prange R. Role for calnexin and N-linked glycosylation in the assembly and secretion of hepatitis B virus middle envelope protein particles. J Virol 1998;72:778-782.

[19] Prange R, Streeck RE. Novel transmembrane topology of the hepatitis B virus envelope proteins. EMBO J 1995;14:247-256.
[20] Sureau C, Fournier-Wirth C, Maurel P. Role of N glycosylation of hepatitis B virus envelope proteins in morphogenesis and infectivity of hepatitis delta virus. J Virol 2003;77:5519-5523.

Table 1. Demographic, clinical and virological data of the patients.

		HBV control group	HBV/HDV case group	p-value
Total patients		n.a.	71	n.a.
Enrolled patients		34	17	n.a.
Female (%) / Male (%)		14 (41.1%) / 20 (58.8%)	7 (41.1%) / 10 (58.8%)	n.s.
Age		37.4 (17 to 54)	38.5 (18 to 58)	n.s.
Cirrhosis		14.7%	52.9%	<0.001
ALT [U/L], median (range)		49 (15 to 172)	48 (20 to 86)	n.s.
HBV viral load [copies/ml], median (range)		$6.8x10^5$ ($3.1x10^3$ -$8.3x10^8$)	$4.6x10^3$ ($1.1x10^3$ -$2.3x10^5$)	<0.001
HBeAg status		negative	negative	n.s.
Viral Genotype	**HBV**	D	D	n.s.
	HDV	n.a.	1	n.a.
Treatment (history)	**IFN**	26%	23%	n.s.
	NAs	62%	59%	n.s.
	no treatment	12%	18%	n.s.

From 71 studied patients with positive HDAg-antibody, 24 had detectable HBV-DNA in serum by real-time PCR. Of this group, 17 cases were successfully amplified and sequenced by in-house PCR. Each case of HBV/HDV-infected patients was matched with two control patients of HBV mono-infected individuals based on the age, sex, viral genotype and HBeAg negativity. Differences were considered significant, if the p-value was <0.05 (chi-square or Mann-Whitney U-test). IFN: interferon, NAs: nucleot(s)ide analogues, n.a.: not applicable, n.s.: not significant.

Figure legends

Fig.1. HBV genome alterations in HBV/HDV infected patients and functional impact of HDV on the replication of HBV polymerase and envelope mutants.

(A) Comparison of amino acid substitution rates (dN/dS) for the S-HBsAg and the rt domain between HBV mono-infected (control, black) and HBV/HDV infected (case, red) patients. (B-C) Huh7 human hepatoma cells were transiently transfected with replication-competent HBV plasmids (5µg, genotype A, subtype adw2). Compared to mono-transfection (S=single, dark gray), the effect of co-transfection with wild type HDV genotype-1 (1µg, D=double, light gray) on HBV replication was studied. Intracellular HBV progeny DNA levels (B, representative Southern Blot after immunoprecipitation for anti-HBc and statistics) and released HBV virions (C, representative Southern Blot and statistics) are shown. Results from n≥3 independent experiments with n=2 biological duplicates each. DL: double-stranded linear form (3.2kb), DM: LAM-resistance double mutation (rtL180M+rtM204V), LAM: lamivudine, pBS: pBluescript (negative control), RC: relaxed circular form (4.0kb), SM: LAM-resistance single mutation (rtM204I), SS: HBV DNA full-length single-stranded form (1.5kb), wt: wild type HBV.

Fig.2. Frequency of mutations in HBV replication/transcription controlling elements, functional impact of HDV on the replication of HBV precore or basal core promoter containing mutants and effects of mutant HBV on HDV replication.

(A) Comparison of HBV nucleotide changes between HBV- (control, dark blue) and HBV/HDV-infected (case, light blue) patients at HBV enhancer domains (Enh I, II)

and transcription control motifs (BOX β, BCP and PC). 'n' represents the number of the patients included in the analyses. (B-E) Huh7 human hepatoma cells were transiently transfected with replication-competent HBV plasmids (5µg, genotype A, subtype adw2) containing different mutations in conjunction with precore (PC) or basal core promoter (BCP) mutations. Compared to mono-transfection (S=single, dark gray), the effect of co-transfection with wild type HDV genotype-1 (1µg, D=double, light gray) on HBV replication was studied. Intracellular HBV progeny DNA levels (B, representative Southern Blot after immunoprecipitation for anti-HBc and statistics) and released HBV virions (C, representative Southern Blot and statistics) are shown. Intracellular HDV genomic RNA levels (D) and extracellular HDV RNA levels in the supernatant (E) of co-transfected cells were assessed by Northern Blot. Results from n≥3 independent experiments with n=2 biological duplicates each. DL: double-stranded linear form (3.2kb), DM: LAM-resistance double mutation (rtL180M+rtM204V), LAM: lamivudine, pBS: pBluescript (negative control), RC: relaxed circular form (4.0kb), SM: LAM-resistance single mutation (rtM204I), SS: HBV DNA full-length single-stranded form (1.5kb), wt: wild type HBV.

235 **Figure 1**

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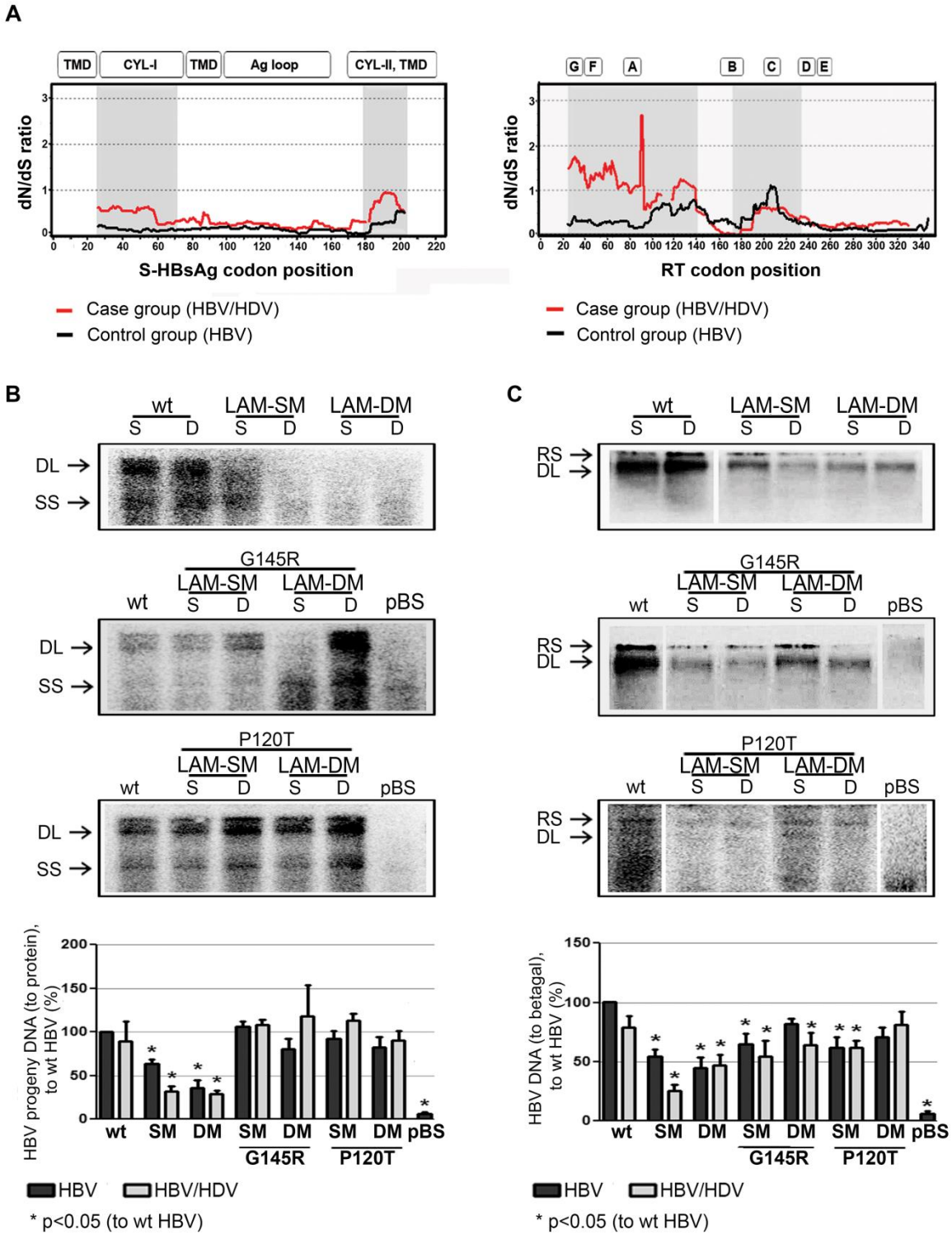

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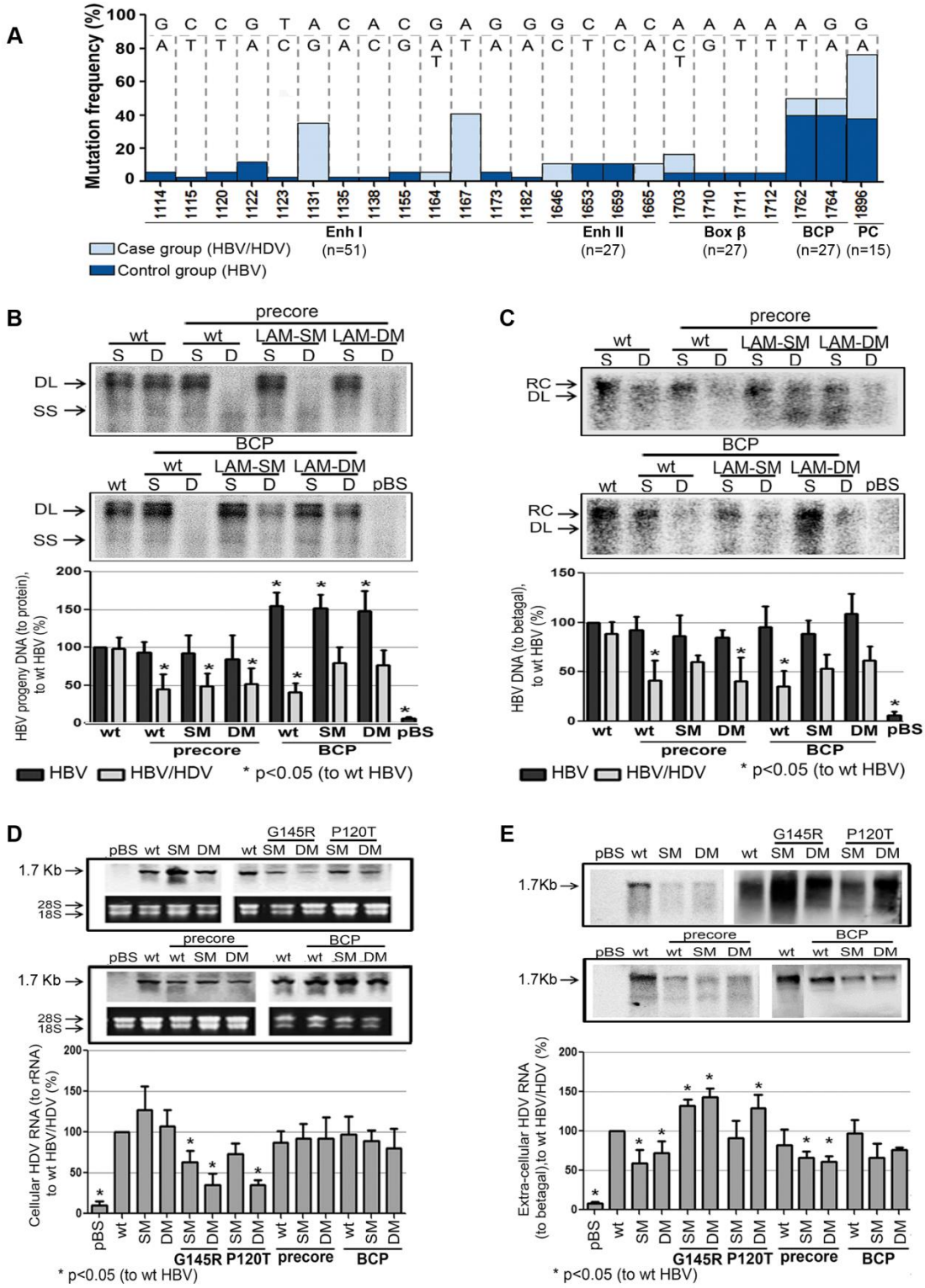
Figure 2


A
Mutation frequency (%)
Case group (HBV/HDV) (n=51)
Control group (HBV)
Enh I
Enh II (n=27)
Box β (n=27)
BCP (n=27)
PC (n=15)
B
precore
BCP
DL
SS
HBV progeny DNA (to protein), to wt HBV (%)
HBV
HBV/HDV
* p<0.05 (to wt HBV)
C
RC
DL
HBV DNA (to betagal), to wt HBV (%)
D
1.7 Kb
28S
18S
Cellular HDV RNA (to rRNA) to wt HBV/HDV (%)
G145R
P120T
E
Extra-cellular HDV RNA (to betagal),to wt HBV/HDV (%)